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Table of Contents

COVER.....	1
SF 298.....	2
Introduction.....	4
BODY.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	7
References.....	8
Appendices.....	9

INTRODUCTION

Chromosome 11q13 is amplified in 13% of primary breast cancers and is indicative of a poor prognosis (1). The region amplified includes the cyclin D1 gene, hst-1, int-2, and ems1 (cortactin). Int-2 and hst-1 are rarely overexpressed in these tumors while both cyclin D1, a cell cycle regulator, and ems1 (also called cortactin), a cytoskeletal protein, are overexpressed. Several studies have shown increased levels of these proteins in different breast cancer cell lines and their amplification is associated with a more invasive phenotype; however, whether or not these proteins are directly contributing to the enhanced tumorigenic potential has not been clearly addressed (2). While the function of cyclin D1 is fairly well understood very little is known about the function of cortactin overexpression in mammary tumorigenesis. The goals of this proposal are to 1) understand the function of cortactin in normal cell growth and differentiation; 2) determine the consequences of cortactin overexpression in mammary epithelial cells; and 3) identify cellular targets of cortactin. We have been using genetic, biochemical, and cell biological approaches including generation of mice carrying a targeted disruption in the cortactin gene and derivation of mammary epithelial cells overexpressing wild type and mutant cortactin.

BODY

The original specific aims were as follows:

- I. To Determine the Function of Cortactin in Normal Cell Growth and Differentiation.**
- II. To Directly Determine the Role of Cortactin on Mammary Epithelial Cell Growth and Differentiation.**
- III. To Identify Proteins Interacting with Cortactin.**

Specific Aim I

- I. To Determine the Function of Cortactin in Normal Cell Growth and Differentiation.**

Previously, we had generated a targeting vector for the cortactin gene. Using this vector we showed that disruption of a single allele of cortactin in the ES cell line, AK7, resulted in an altered cell phenotype. ES cells which are heterozygous for the cortactin gene, differentiate into a population of cells of unknown origin. Not surprisingly, these cells are unable to contribute significantly to the germline. As reported last year, we have used two different ES cell line, to target the cortactin locus. J1 or TC1 ES cells which are heterozygous for cortactin, do not differentiate. We injected 4 TC1 clones and 2 J1 clones and obtained chimeras. One of the J1 clones resulted in 80% chimerism while poor results were obtained with the TC1 clones. The 80% chimera was bred to obtain germline transmission. Only a single litter was born and with only three pups surviving until weaning. None of these were agouti, indicating that these were not heterozygous for cortactin. Subsequent attempts to mate this male failed and the animal died by four months of age. While it is difficult to draw any firm conclusions from this result, based on the previous findings in the AK7 ES cells, and the absence of any problems with other mice in our colony, we speculate that this may be additional evidence for a dosage sensitive phenotype.

Based on these findings, we are currently trying to generate a targeting vector to make a conditional mutation in the cortactin locus. We hope to have this vector and the initially targeted ES cells by the end of the year.

As an alternate strategy to understanding cortactin function, we are using RNA interference (RNAi) to block cortactin function(). This strategy has been used to block expression of a number of different cytoskeletal genes in mammalian cells, including Arp2/3, zyxin, and vinculin. We have designed two different oligomers and are using the vector based strategy for stable expression (). We are initially testing two different cell lines. Hela cells and MCF10A cells. Hela cells are being used since this line has been used successfully to target many different genes, so it will be a good system to test our potential cortactin siRNAs. Ultimately, however, we would like to address cortactin function in mammary cells, so MCF10A cells are being used for this purpose. RNAi has been used successfully in this line to knockdown expression of an exchange factor, Vav-2 (JS Brugge, personal communication). The use of both this in vitro blocking strategy and the longer term in vivo work in the mice, should define cortactin's function in normal cell growth and differentiation.

Specific Aim II

II. To Directly Determine the Role of Cortactin on Mammary Epithelial Cell Growth and Differentiation.

One of the major goals of this proposal was to determine whether overexpression of cortactin or variants of cortactin affected mammary epithelial biology. To this end we have used an immortalized cell line, Scp2, which can mimic some of the morphogenetic events that occur in vivo (3). When placed in an appropriate extracellular matrix in the presence of lactogenic hormones, these cells form a polarized three-dimensional luminal structure that is capable of secreting milk proteins into the lumen. Using these cells we have shown that expression of full length cortactin results in a number of phenotypic changes compared to control cells. These include a "scattered" cell morphology, an increase in cell migration, decreased milk production, and alterations in polarity. Cells expressing full length cortactin have an altered cell morphology. While control cells form tight epithelial colonies, cortactin expressing cells are more refractile. This is typical of cells that are more migratory. Consistent with this phenotype, these cells are able to migrate and close a wound faster than the control cells. The cells, however, do not appear to be more invasive. Since invasion is likely to be a late event, we may look at the consequences of overexpression in tumorigenic lines such as T47D cells.

While cortactin does not induce SCp2 cells to become invasive, it does affect the differentiation program of these cells. When the cells are plated in matrigel and given lactogenic hormones, they organize themselves into alveolar like structures and produce milk proteins such as b-casein. Expression of cortactin resulted in a dramatic decrease in b-casein positive cells. In addition, when we analyzed the organization of the alveolar structure, there was a loss of polarity as judged by a loss of E-cadherin and b-catenin localization and a dramatic decrease in the junctional protein ZO-1. We have examined whether this phenotype is also seen in human mammary cells using the MCF10A cells. The results have been somewhat variable, but we think this is due to levels of expression. Currently, it appears that if the levels of overexpression are adequate, then cortactin can induce a scattering phenotype in MCF10A cells. The early results with differentiation suggest that it is also capable of disrupting differentiation. If these results hold, then we may switch our studies completely to the MCF10A system. This is being done since the SCp2 cells, while extremely useful, have been more difficult to work with and maintain. In addition, it may be more relevant to use human cells, where possible, for these studies.

To determine what regions of cortactin may be important for this phenotype, a number of mutants were made which were tagged with a HA epitope. Originally we had hoped that expression of the N-terminal half or c-terminal half may in some way interfere with cortactin function. However, our results suggest that expression of the full length, N-terminal or c-terminal half of cortactin in SCp2 results in essentially the same phenotype. This raises the possibility that these two major halves of cortactin are able to either signal independently to induce these changes or alternatively, can activate the endogenous cortactin protein. The N-terminus localizes to the cortical cytoskeleton where the wildtype protein is found; however, the C-terminus only weakly localizes to the cortical cytoskeleton and in 95% of the cells shows cytoplasmic staining. These results suggest that the localization of the protein to the cortical cytoskeleton is not required for inducing these phenotypes. We are currently working to localize these proteins in the differentiated structures. We have had some technical problems due to nonspecific staining with the HA antibody when the cells are in matrigel. If this continues to be problematic, we can address this question using our GFP-tagged cortactin variants, which behave identical to the HA constructs in the monolayer and differentiation assays.

Given that the N-terminal and C-terminal halves of cortactin can alter the properties of SCp2 cells and MCF10a cells, we have made additional mutations to see if we can suppress these phenotypes. The N-terminal half of cortactin contains a repeat region which can bind f-actin (4). The fourth repeat has been shown to bind to phosphatidylinositol lipids which have been implicated in regulating cell migration (5). Cells expressing this mutant no longer affect the migratory or differentiation properties of the SCP2 cells. Similarly disruption of the Src Homology 3 (SH3) domain in the c-terminal half of cortactin also blocks the ability of the c-terminal half to alter SCP2 cells. Thus, these regions may somehow contribute to the deregulation of cell migration and/or differentiation in mammary epithelial cells and potentially be activating pathways normally regulated by cortactin. We are currently trying to wrap up these studies and prepare a manuscript. Based on this work, we plan to take these studies in vivo by making MMTV-cortactin transgenic mice. These mice should express cortactin in the mammary gland. The construct has been made. These studies should provide an interesting in vivo model for looking at the consequences of cortactin overexpression. Studies with these animals will include crosses with different mammary tumor models to determine whether cortactin overexpression makes the tumors more metastatic.

Finally, we have also begun to look at potential cooperativity between cortactin and two other genes in the locus, cyclin D1 and Gab2. As indicated above, cyclin D1 is another gene in this locus which also has elevated protein levels. Our analysis in MCF10A cells indicates that overexpression of cortactin along with cyclin D1 appears to dramatically affect mammary differentiation resulting in disorganized structures. We plan to use both mutants of cortactin and mutants of cyclin D1 to understand this phenotype. We also are looking at potential cooperativity between a second gene, Gab2, and cortactin. This work is being done in collaboration with Dr. Haihua Gu and Dr. Benjamin Neel's laboratories.

Specific Aim III

III. To Identify Proteins Interacting with Cortactin.

The third major goal of this proposal was to identify proteins interacting with cortactin. We have used a number of approaches including yeast two hybrid screens and small cDNA pool screens. In addition, we have taken advantage of the identification of the optimal binding sequence of the SH3 domain and used a bioinformatics approach. This has yielded the identification of a multi-domain Rho family GTPase exchange factor, facio-genital dysplasia

gene 1 (FGD1) as a binding protein for cortactin. Given that expression of cortactin enhances cell migration and that its localization is known to be regulated by a Rho family member, Rac, FGD1 is likely to be an important effector/regulator of cortactin function (6). We have obtained the FGD1 cDNA and are currently testing its role in cortactin biology. A second protein that has been identified is the Wiskott-Aldrich syndrome interacting protein, Wip, another regulator of the actin cytoskeleton (7). In collaborative study with Raif Geha's laboratory at Children's hospital/Harvard Medical school, we have shown that wip binds to the cortactin SH3 domain. Two other proteins include an Arf GAP called, Def-1 and as indicated above an adaptor protein, Gab2. In collaboration with Dr. Tom Robert's lab, we will examine Def-1's role in the cortactin phenotype.

KEY ACCOMPLISHMENTS

- λ Generation of ES cell lines which are heterozygous and potentially homozygous for the cortactin gene.
- λ Generation of high percentage chimeras from these ES cells
- λ Discovery that expression of cortactin and different variants of cortactin can alter the properties of mammary epithelial cells and that cortactin can cooperate with cyclin D1 to dramatically disrupt mammary differentiation.
- λ Generation of a MMTV-Cortactin vector for transgenic studies.
- Identification of FGD1 and Wip as cortactin binding proteins.

REPORTABLE OUTCOMES

Manuscripts:	None. We are in the process of writing up the work described in specific Aim II
Presentations:	University of North Carolina, Cell and Developmental Biology Dept. (no abstract available)
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CONCLUSIONS

We have made progress on all three specific aims. Over the next year we hope to make considerable progress towards understanding the role of cortactin in vivo by generating a conditional knockout of cortactin. We will also generate a MMTV-cortactin transgenic. We will continue to pursue our in vitro studies in the mammary epithelial cell lines and by looking at additional cortactin mutants, known regulators and effectors, and looking at interacting proteins identified in specific aim III, we should gain a better understanding of how expression of cortactin contributes to mammary tumor progression. Finally, the data obtained thus far, should provide us with the necessary preliminary information needed to pursue additional funding for these studies.

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APPENDICES.

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